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**Antimicrobial Activity of Chitosan Against *Candida krusei*,
Saccharomyces cerevisiae, and *Zygosaccharomyces bailii***

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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College: Agriculture Department: Food Science & Technology

Faculty Mentor: Dr. Svetlana Zivanovic

PROJECT TITLE: Antimicrobial Activity of Chitosan Against
Candida krusei, Saccharomyces cerevisiae, and
Zygosaccharomyces bailii

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Svetlana Zivanovic Faculty Mentor

Date: 05-03-2004

Comments (Optional):

Mechelle Elosiebo

**Antimicrobial Activity to Chitosan Against *Candida krusei*, *Saccharomyces cerevisiae*,
and *Zygosaccharomyces bailii***

Honor Research Project

Honors Program (Senior Project)

The University of Tennessee, Knoxville

May 3, 2004

Abstract

Chitosan is a positively charged natural polysaccharide. Due to its strong antimicrobial effects, metal, protein and lipid binding properties, and thickening and film forming capabilities it has potentials to be used in the food industry, agriculture, and medicine. The objective of this research was to determine the antimicrobial effect of chitosans with different molecular weight towards three yeast species: *Candida krusei*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii*. The experiment was performed with medium molecular weight (450 kDa) and oligosaccharide chitosan (degree of polymerization 15) applied in the final concentrations of 0 %, 0.005 %, 0.010 %, and 0.050 % in apple juice and acetic acid solution. All samples and controls were inoculated with 10^4 cfu/ml and incubated at 22 °C with constant shaking. Microbial growth was evaluated for seven days by measuring optical density (OD) at 600 nm and by plate counting on yeast and mold agar. Results showed that MMW chitosan was more effective as an antimicrobial agent than OL chitosan. *C. krusei* was most resistant while *Z. bailii* was most sensitive to chitosan. The minimum tested concentration, 0.005%, delayed growth of all tested species for minimum 24 hr, while 0.050% MMW chitosan delayed growth in *C. krusei* and *S. cerevisiae* for 4, and *Z. bailii* for 6 days. These results indicate that chitosan could be used as an effective antimicrobial agent in the food industry.

Introduction

Chitosan is commercially produced by deacetylation of chitin, a β -(1,4)-D-linked polymer of N-acetylglucosamine, extracted from crustacean shells (Roller 2000, Rhoades 2000, No, 2002). It has recently gained the attention of the scientific community due to its functional properties that have great potential for application in agriculture, medicine, and the food industry. Its applications are based on film-forming capabilities, mineral-binding properties, hypolipidemic activity, biodegradability, antimicrobial activity, immunoadjuvant activity, acceleration of wound healing, and eliciting of phytoalexins (Roller 1999). One of its main potentials in the food industry is its use as a natural, non-toxic, biodegradable alternative to synthetic preservatives.

Antimicrobial properties of chitosan have been extensively researched (Roller 1999). At pH < 6 chitosan is positively charged and readily reacts with negatively charged particles, including bacterial cells and metals ions (Li et al.1997). Chitosan has been used as a chelator of toxic metals, as a moisturizer in hair care and personal care products, and as a cholesterol level reducer in rats and a limited number of humans (Roller 1999). The majority of chitosan antimicrobial research to date has investigated its effects on bacteria. Sudarshan et al. (1992) demonstrated its ability to inactivate bacteria between one and five log cycles within one hour. Furthermore, it has been shown that chitosan equal inactivate both gram-negative and gram-positive organisms, signifying a non-specific mode of attack (Roller, 2000).

Although there has been a focus of investigation into bacteriocidal effects, there is a relative lack of information of chitosan activity against fungal species. The principle damage caused by spoilage yeasts is in their CO₂ production during fermentation that causes bloating in packages and undesired flavor of the food product. These negative effects can be prevented

by addition of chemicals such as sorbate or propionate, but consumers generally desire no presence of such chemicals in their foods (Savard, 2002). In recent years, the need to identify natural products that can inhibit the growth of unwanted yeasts has been increased. The research on chitosan's antimicrobial potential against food spoilage yeast in apple juice may result in novel ways to extend the product safety and shelf life. Understanding the exact inhibition mechanism and determination of minimal inhibitory concentrations will help in designing practical applications of chitosan as an affective antimicrobial agent in various food products.

The objectives of this research were to determine the effect of molecular weight on the antimicrobial potential of chitosan, and to determine the susceptibility of three species of food spoilage yeasts to chitosan.

Materials and Methods

Medium molecular weight chitosan (MMW, 450 kDa) and chitosan oligosaccharide (OL, 3 kDa) were obtained from Sigma (St. Louis, MO). Pasteurized apple juice with no additives was purchased in a local grocery store. Chitosan stock solutions were prepared by dissolving 2 % w/v chitosan in 1 % v/v acetic acid using pasteurized apple juice as a solvent. The solution was thermally sterilized by autoclaving at 121 °C for 15 minutes and kept at room temperature until use but not longer than 7 days. Three yeast species, *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae*, and *Candida krusei* from the Department of Food Science and Technology at The University of Tennessee culture collections, were used as test microorganisms. Yeast species were sub-cultured in yeast and mold broth (YMB) and transferred at least three times before a 62-hr old culture was used for

inoculation. The test media was pasteurized apple juice with 1 % acetic acid. The addition of different volumes of chitosan stock solution resulted in various levels of chitosan (0.005 %, 0.01 %, and 0.05 %) while keeping constant concentration of the acid. Two controls were used in the experiment: pure, pasteurized apple juice and apple juice with 1 % acetic acid. Samples and controls were inoculated with 10^4 cfu/ml juice and incubated for 7 days at 22 °C with constant shaking (100 rpm; Gyrotory Shaker 62, New Brunswick Scientific Co., Inc., Edison, NJ). The yeasts species were inoculated in triplicates for all chitosan concentrations. Microbial growth was determined by measuring optical density at 600 nm using UV-VIS spectrophotometer (Unicam UV-1, Cambridge, UK). The microbial growth was determined against pasteurized and un-inoculated apple juice and read every 12 hours for the first two days and every 24 hours for the duration of 7 days.

Results and Discussion

Chitosan showed significant antimicrobial activity against *S. cerevisiae*, *C. krusei*, and *Z. bailii*. *Z. bailii* was the most susceptible to chitosan and its growth was completely inhibited by 0.05% MMW chitosan for 6 days, only showing positive growth on seventh day (Figure 1). *C. krusei* showed the highest level of resistance and its growth was inhibited for maximum of four days (Figure 2). *S. cerevisiae* susceptibility appeared to be between the other two yeasts. It failed to grow in the presence of 0.01 % or higher concentrations of MMW chitosan for four days but lower levels did not have inhibitory effects (Figure 3). MMW chitosan in concentration of 0.05 % showed the greatest antimicrobial activity against all strains of yeast tested. It delayed growth of *C. krusei* and *S. cerevisiae* for 4, and of *Z. bailii* for 6 days. Medium molecular weight chitosan was more effective as an antimicrobial

agent than oligosaccharide (Figures 1-3). Although higher concentrations and molecular weights were more effective in inhibiting yeast growth, all tested concentrations of chitosan slowed yeast growth. The minimum tested concentration of medium molecular weight chitosan (0.005 %) delayed growth of all tested species for minimum of 24 hours while 0.005% chitosan oligosaccharide delayed growth of all species for a minimum of 12 hours. Chitosan oligosaccharide in concentration of 0.05 % completely inhibited *Z. bailii* and *S. cerevisiae* for 48 hours but caused only 1 log reduction for *C. krusei*. At the same concentration, medium molecular weight chitosan delayed spoilage for at least 3 days, regardless on the yeast species.

Correlating with the fact that the smaller sized chitosan was more effective in fungal growth inhibition in this experiment, Savard et al.(2002) demonstrated that the most highly degraded chitosan in his experiment was the most effective microbial growth inhibitor against three lactic acid bacteria and yeasts including *Saccharomyces unisporus* and *Saccharomyces bayanus*. Savard et al. (2002) concludes that partly hydrolyzed chitosan shows potential for preservation in acidic foods and that the degree of inhibitory properties depends on the degree of hydrolysis. The maximal inhibitory abilities of smaller sized chitosan particles is confirmed also by Kendra and Hadwiger (1984). In this experiment, the shortest oligomer demonstrated the highest antifungal activity. Although lower weight chitosan was not clearly defined, experiments suggested that in an agar based media, lower weight chitosan was more effective than high molecular weight chitosan in inhibiting range of phytopathogenic fungi (Hirano and Nagao 1989). Hirano and Nagao (1989) also showed that chitosan's effectiveness in inhibiting microbial growth is maximized as size decreases to a certain point. After chitosan has reached this critical size, its microbiocidal activity is lessened. Therefore, after a certain reduction in

size, chitosan's antimicrobial activity decreases. No (2002) showed that in general, chitosans had greater antimicrobial activity than chitosan oligomers. Uchida (1989) found that while chitosan had effective antimicrobial abilities against bacteria, chitosan oligomers' antimicrobial abilities were weaker or non-existent. The sensitivity of *Z. bailii* in our experiment parallels the results in experiments of Rhoades and Roller (2000) where 0.1 g of lysozyme-degraded chitosan per liter completely inhibited *Z. bailii* growth for 4 days. The authors also found that the effectiveness of chitosan against *Candida sp.* results from exposure to chitosan which either kills the cells or renders them nonculturable in a short amount of time. The results of this experiment align with many trends in current literature such as the fact that larger sized chitosan is more effective.

Many hypotheses have been proposed to explain the mechanism of antimicrobial effects of chitosan. One of hypotheses is that the mechanism involves interactions of chitosan positively charged molecules with negatively charged constituents of microbial cell walls and membranes interrupting normal cell metabolism (Savard, 2002). Several authors indicated that chitosan may directly affect cell membrane function (Leuba and Stossel, 1986). Leuba and Stossel (1986) showed that chitosan caused proteinous UV-absorbing materials to leak from cell membranes of *Pythium paroecandrum*, a plant pathogen. Chitosan can chelate metal ions and it is possible that chitosan can inhibit fungal growth by chelating metals such as calcium and iron, which are essential for cell reproduction (Roller 1999) Furthermore, binding and flocculation of proteins by chitosan is pH dependent. In acidic environments, such as in apple juice (pH~ 3.4), chitosan molecules are highly protonated and may interact with the cell membrane proteins causing their denaturation, damage of membrane functions, and death of microbial cell. However, inhibition of microbial growth by chitosan depends on its

concentration, molecular weight, degree of acetylation, target microorganism, media composition, and temperature (Savard, 2002). Roller and Covill (1999) showed that chitosan's antimicrobial activity against yeasts is biocidal and reduced the eight yeasts that they examined "by up to three log cycles within the first 5 hours of exposure." Allan and Hadwiger (1979) studied the effect of 46 fungal strains and concluded that the natural presence of chitosan in cell walls of some fungi enables these strains to be more resistant to added chitosan. This may explain the resistance of *C. krusei* to chitosan.

Conclusion

The data showed that presence of chitosan at levels as low as 0.005% can significantly inhibit the growth of spoilage yeasts: *Z. bailii*, *S. cerevisiae*, and *C. krusei*. Both tested chitosans showed significant antimicrobial capabilities, but chitosan of higher molecular weight (450 kDa) showed stronger antimicrobial activity than chitosan oligomer (3 kDa). Medium molecular weight chitosan in concentration of 0.05% expressed the most effective inhibition of yeast growth. These results indicate that chitosan could be used in the food industry as an effective antimicrobial agent.

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Figure captioning

Figure 1: Antimicrobial activity of medium molecular weight chitosan (MMW) and chitosan oligosaccharide (OL) against *Zygosaccharomyces bailii* grown in apple juice (AJ) at 22 °C.

Figure 2: Antimicrobial activity of medium molecular weight chitosan (MMW) and chitosan oligosaccharide (OL) against *Candida krusei* grown in apple juice (AJ) at 22 °C.

Figure 3: Antimicrobial activity of medium molecular weight chitosan (MMW) and chitosan oligosaccharide (OL) against *Saccharomyces cerevisiae* grown in apple juice (AJ) at 22 °C.

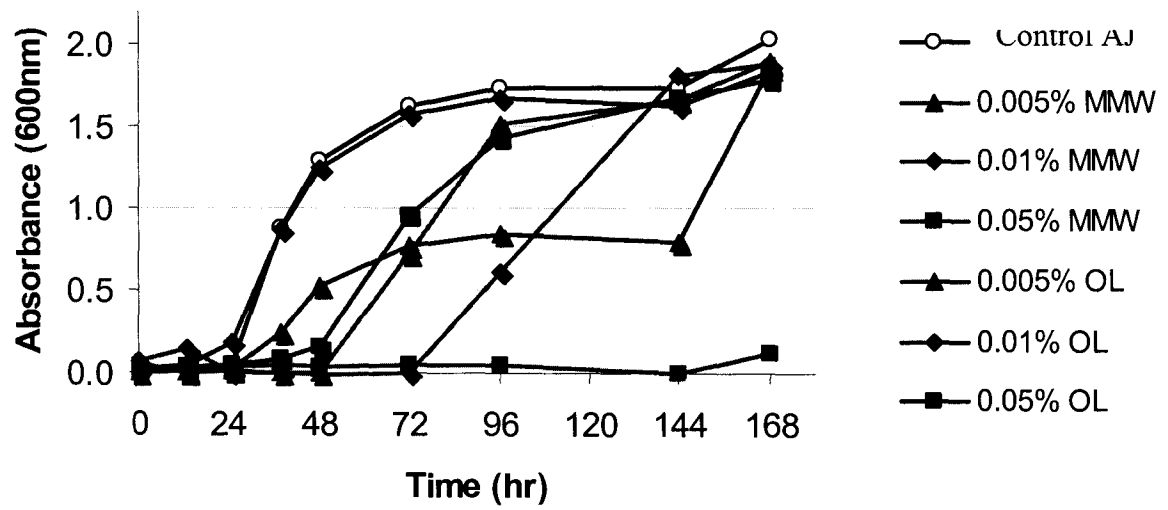


Figure 1

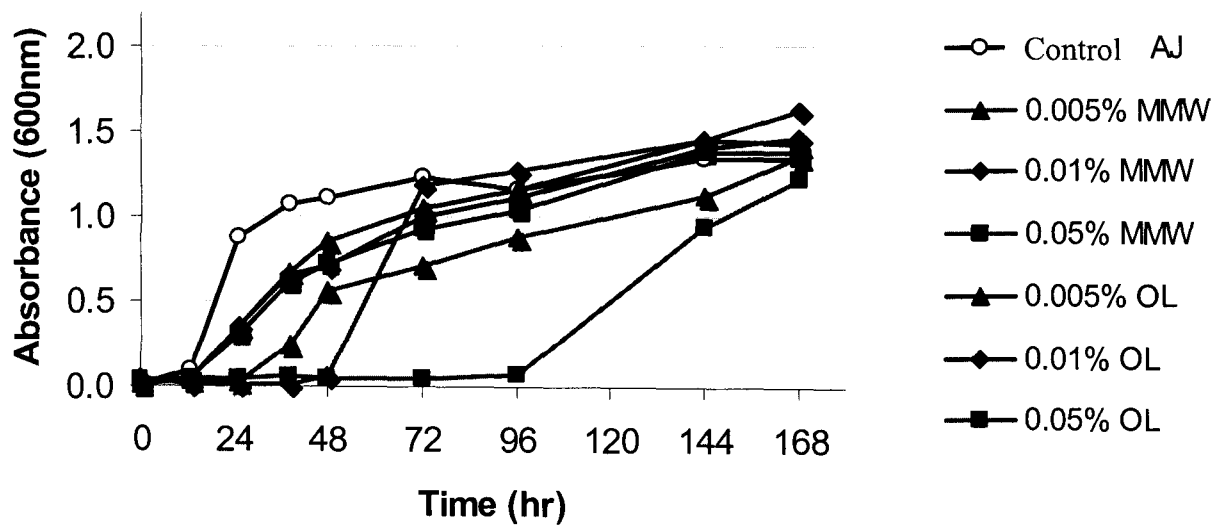


Figure 2

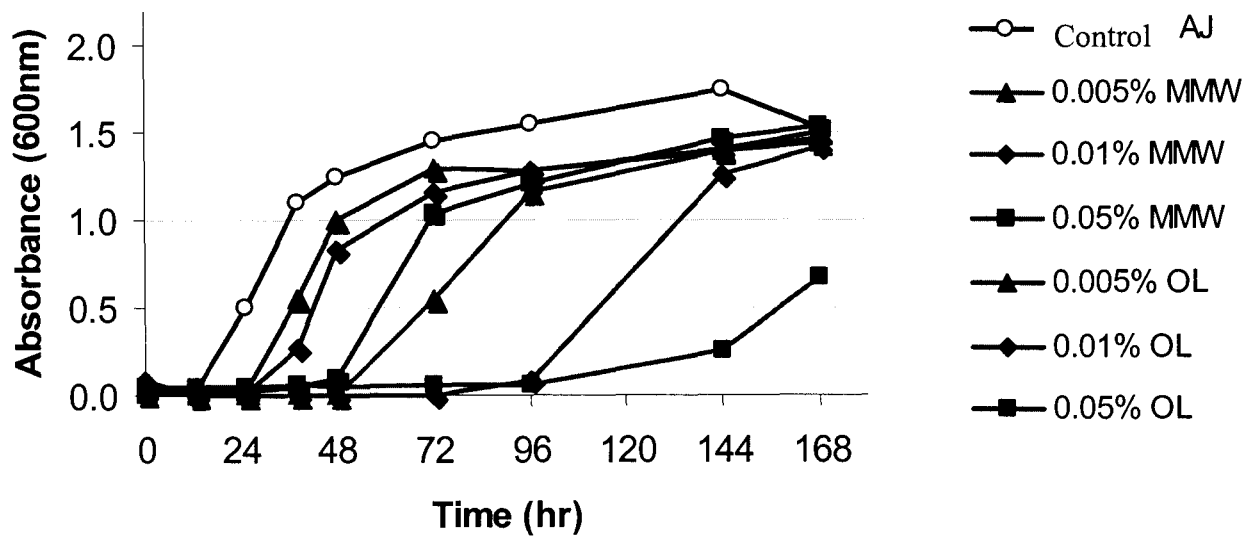


Figure 3